In Vitro Analysis of the Origin and Maintenance of O-2A^{adult} Progenitor Cells

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Abstract. We have been studying the differing characteristics of oligodendrocyte-type-2 astrocyte (O-2A) progenitors isolated from optic nerves of perinatal and adult rats. These two cell types display striking differences in their in vitro phenotypes. In addition, the O-2A^{perinatal} progenitor population appears to have a limited lifespan in vivo, while O-2A^{adult} progenitors appear to be maintained throughout life. O-2A^{perinatal} progenitors seem to have largely disappeared from the optic nerve by 1 mo after birth, and are not detectable in cultures derived from optic nerves of adult rats. In contrast, O-2A^{adult} progenitors can first be isolated from optic nerves of 7-d-old rats and are still present in optic nerves of 1-yr-old rats. These observations raise two questions: (*a*) From what source do O-2A^{adult} progenitors originate; and (*b*) how is the O-2A^{adult} progenitor population maintained in the nerve throughout life?

We now provide in vitro evidence indicating that O-2A^{adult} progenitors are derived directly from a subpopulation of O-2A^{perinatal} progenitors. We also provide evidence indicating that O-2A^{adult} progenitors are capable of prolonged self renewal in vitro. In addition, our data suggests that the in vitro generation of oligodendrocytes from O-2A^{adult} progenitors occurs primarily through asymmetric division and differentiation, in contrast with the self-extinguishing pattern of symmetric division and differentiation displayed by O-2A^{perinatal} progenitors in vitro. We suggest that O-2A^{adult} progenitors express at least some properties of stem cells and thus may be able to support the generation of both differentiated progeny cells as well as their own continued replenishment throughout adult life.

For at least several different cellular lineages, substantial differences appear to exist between the precursor cells which function during development and those which are present in the adult animal. For example, there are fundamental differences between embryonic myoblasts and adult satellite muscle cells (Cossu et al., 1987; Schafer et al., 1987), and between fetal and adult cells of both the haematopoietic and sympathoadrenal lineages (Anderson 1989; Wood et al., 1985). In addition, optic nerves of both perinatal and adult rats contain progenitor cells which can be induced to differentiate in vitro into either oligodendrocytes or type 2 astrocytes (Raff et al., 1983*b*; ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989). However, the oligodendrocyte type 2 astrocyte (O-2A)[1] progenitors isolated from optic nerves of adult rats differ from their perinatal counterparts in antigenic phenotype, morphology, cell-cycle time, motility, and time course of differentiation in vitro (Wolswijk and Noble, 1989).

The appearance of adult-specific precursors in any lineage raises questions about their developmental origin. Are these cells derived from a common ancestor cell which, for example, initially gives rise to O-2A^{perinatal} progenitors, but then gives rise to O-2A^{adult} progenitors during later stages of development? Alternatively, are perinatal and adult precursor populations derived from two distinct ancestors, despite being specialized to produce similar terminally differentiated end-stage cells?

The continued presence of precursor populations in adult animals also raises questions about how such populations are maintained within any particular tissue throughout life. The maintenance of a precursor population throughout life is generally thought to be associated with the presence of a stem cell population which supplies new cells to the precursor pool for use in cell replacement following normal turnover or injury. For example, it has been suggested that the presence of proliferating O-2A progenitors in the adult animal requires the existence of a pre-progenitor, or stem cell, compartment in the O-2A lineage (ffrench-Constant and Raff, 1986). The requirement for a stem cell compartment to support the prolonged maintenance of dividing O-2A progenitors in the nerve is further indicated by the self-extinguishing behavior of O-2A^{perinatal} progenitors: O-2A^{perinatal} progenitors grown in vitro in the presence of purified cortical astrocytes (as a source of mitogen; Noble and Murray, 1984; Noble et al., 1988) tend to divide and differentiate symmetrically, such that clonally related cells synchronously differentiate into oligodendrocytes within a limited number of divi-

1. *Abbreviations used in this paper*: GFAP, glial fibrillary acidic protein; O-2A, oligodendrocyte-type-2 astrocyte.

sions (Temple and Raff, 1986; Raff et al., 1988). In addition, O-2A^{perinatal} progenitors are present only in small numbers in cultures prepared from optic nerves of 1-mo-old rats (Wolswijk et al., 1990) and are not detectable in cultures prepared from optic nerves of adult rats (Wolswijk and Noble, 1989). Thus, the properties of O-2A^{perinatal} progenitors seem to be inconsistent with the maintenance of a proliferating O-2A progenitor population throughout life.

In the present studies we present data suggesting that the most likely ancestors of O-2A^{adult} progenitors are a subset of O-2A^{perinatal} progenitors, thus indicating that the O-2A^{perinatal} progenitor population is tripotential in vitro, rather than bipotential. We also show that members of the O-2A lineage have the capacity for extended self-renewal in vitro, that such self-renewal is associated with the conversion of populations with a *perinatal* phenotype to populations with an *adult* phenotype and that dividing O-2A^{adult} progenitors isolated from adult rats appear to generate oligodendrocytes through a process of asymmetric division and differentiation. Thus, O-2A^{adult} progenitors may be responsible for their own self renewal and in this, and other respects, seem likely to represent a population of glial stem cells in adult animals.

Materials and Methods

Cell-type Preparation and Identification

All O-2A progenitors were grown in DME containing defined additives (DME-BS, modified from Bottenstein and Sato, 1979; as described in Wolswijk and Noble, 1989).

To induce differentiation of O-2A progenitors into type 2 astrocytes, cells were grown in DME containing 10% FCS (DME-FCS; Raff et al., 1983*b*; Wolswijk and Noble, 1989).

Purified cortical astrocytes were prepared from cerebral cortices of newborn rats as described previously (Noble et al., 1984; Noble and Murray, 1984).

All antibodies used for cell-type identification, and all staining methods, have been described previously (Raff et al., 1983; Noble and Murray, 1984; Wolswijk and Noble, 1989). In brief, cell types are defined as follows: O-2A^{perinatal} and O-2A^{adult} progenitors are labeled with the A2B5 monoclonal antibody (Eisenbarth et al., 1979), but not with antibodies against galactocerebroside (GalC, an oligodendrocyte-specific glycolipid; Raff et al., 1978; Ranscht et al., 1982) or antisera directed against glial fibrillary acidic protein (GFAP, an astrocyte-specific cytoskeletal component; Bignami et al., 1972). O-2A^{adult} progenitors are labeled also with the O4 mAb (Sommer and Schachner, 1981; Wolswijk and Noble, 1989), which labels only a subpopulation of O-2A^{perinatal} progenitors (Sommer et al., manuscript submitted for publication). Oligodendrocytes are GalC$^+$, type 2 astrocytes are A2B5$^+$GFAP$^+$ (Raff et al., 1983*a*) and type 1 astrocytes are A2B5$^-$GFAP$^+$ (Raff et al., 1983*a*). All fluorescein- and rhodamine-conjugated second layer antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL) and were used at a dilution of 1:100. Anti-GalC and A2B5 antibodies were applied to living cultures, while anti-GFAP antiserum was applied to cultures following fixation in methanol (−20°C, 10 min). These antibodies were used in various combinations for two fluorochrome immunofluorescence analysis as described previously (e.g., Raff et al., 1983*b*; Noble and Murray, 1984; Wolswijk and Noble, 1989). Following labeling for immunofluorescence any unfixed cultures were fixed in methanol (−20°C, 10 min), placed cells down in a drop of Citifluor to retard fading of fluorescein (Johnson et al., 1982) and sealed with nail varnish. Cultures were viewed on a Zeiss Axiphot microscope (Zeiss, Oberkochen, Germany) equipped with phase contrast and epi-UV illumination and selective filters for fluorescein and rhodamine.

Time-lapse Microcinematography

O-2A progenitor cells derived from optic nerves of 21-d-old rats were grown in poly-L-lysine coated petri dishes, of which the edges had been coated previously with 250,000 purified cortical astrocytes to supply the continuous presence of mitogens in the medium. Cultures were then followed using time-lapse microcinematography (Riddle, 1979) for periods of up to 8 d (as in Small et al., 1987; Wolswijk and Noble, 1989; Wolswijk et al., 1990). Recording started 1 d after plating and fields were chosen that contained at least three O-2A progenitor-like cells. Families analyzed in detail had to meet the criteria that the majority of clonally related cells remained within the field of view (0.77 mm × 1.08 mm), cells were not overgrown by dividing flat cells (i.e., non O-2A lineage cells), and cells in the colonies appeared healthy until the end of filming.

Cells were analyzed on the basis of morphology, rate of migration and cell-cycle length. Cells were divided into categories of bipolar, pseudo unipolar, and multipolar. Analysis of migration rates was carried out by dividing the distance migrated by a cell between two cell divisions by its cell-cycle length. Intermitotic times were determined by analysis of frame number. Our previous studies (Small et al., 1987; Wolswijk and Noble, 1989; Wolswijk et al., 1990, 1991) have indicated that, in optic nerve cultures, the different cell types of interest express predominantly the following characteristics when grown in the presence of purified cortical astrocytes: O-2A^{perinatal} progenitor = bipolar, short cell-cycle time (18 ± 4 h), rapid rate of migration (21 ± 2 μm/h); O-2A^{adult} progenitor = pseudo-unipolar, long cell cycle time (65 ± 18 h), slow rate of migration (4 ± 1 μm/h); oligodendrocyte = multipolar, non-dividing, and non-migratory.

Serial Passaging

Perinatal optic nerve cells were serially passaged for up to six passages (over the course of 3 mo) on monolayers of purified and irradiated cortical astrocytes (to promote progenitor division; see Noble and Murray, 1984; Wolswijk and Noble, 1989). In two experiments we passaged optic nerve cells from newborn rats, which contain no detectable O-2A^{adult} progenitors (Wolswijk et al., 1990). We also separately passaged cells from optic nerves of 7-d-old rats, in which ≤2% of the O-2A progenitors appear to be *adult*-like (as defined by antigen expression and morphology; Wolswijk et al., 1990); in the latter case, the suspensions of optic nerve cells were treated first with the O4 mAb (Sommer and Schachner, 1981) and complement to lyse adult-like progenitors (which are O4$^+$; see Wolswijk and Noble, 1989; Wolswijk et al., 1990; and Table I); such treatment would also have lysed O-2A^{perinatal} progenitors cells with an O4$^+$ antigenic phenotype, which represents a later stage of antigenic differentiation than that expressed by O-2A^{perinatal} progenitors isolated from embryonic animals (Levi et al., 1987; Sommer et al., submitted for publication). Complement-mediated lysis was carried out by incubating freshly isolated cells with sterile undiluted O4 hybridoma supernatant for 30 min at 37°C. Cells were then washed through 10 ml of DME, followed by addition of a 1:10 dilution of agarose-adsorbed rabbit complement (e.g., Noble and Murray, 1984; Wolswijk and Noble, 1989) for 30 min at 37°C. Cells were washed again and plated directly on monolayers of purified cortical astrocytes, as described below. Preparations from newborn and 7-d-old animals yielded similar results and were therefore combined in Fig. 3; in addition, four partial repeats of the serial passaging experiments gave similar results.

Passaging experiments were initiated by plating 5,000 optic nerve cells onto irradiated monolayers of cortical astrocytes growing in 25 cm^2 tissue culture flasks (Falcon). Cells were grown for 10-14 days in DME-BS + 0.5% FCS, with half the medium being replaced every 2 d. This growth protocol was required to promote the continued health of the optic nerve cells for 3 mo of serial passaging. After 10–14 d, cells were rinsed in $Ca^{2+}Mg^{2+}$-free DME, followed by an incubation in 5 ml of $Ca^{2+}Mg^{2+}$-free DME containing 0.54 mM EDTA and 3,000 U/ml of trypsin. During incubation, flasks were observed under an inverted microscope. When the cells growing on top of the monolayer (i.e., the O-2A lineage cells) began to round up (which occurred before disaggregation of the astrocyte monolayer) the top cells were detached by a sharp tap on the side of the flask. Trypsinization was halted by addition of 1 ml of DME containing 5,200 U/ml of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), 74 U/ml of DNAse 1 (Sigma Chemical Co.) and 3.0 mg/ml BSA (fraction V; Sigma Chemical Co.). Cells collected from the flask were gently triturated through a 5-ml pipette, centrifuged (5 min, 500 *g*) and resuspended in DME-BS/0.5% FCS. As noted in the text, in some experiments oligodendrocytes were removed using complement mediated cytolysis with anti-GalC antibody. In such experiments, lysis was achieved by treating cells with sterile anti-GalC mAb after removal from the flask, followed by a rinse and addition of complement. Before replating, cells were rinsed again. Cells were then replated into fresh flasks, or onto coverslips, both containing irradiated monolayers of cortical astrocytes. The coverslips were kept on raised platforms overnight to allow the cells to adhere, transferred to Falcon six-well trays (three coverslips/well) and grown in DME-BS/0.5% FCS. The cells

obtained from one flask were plated onto 30 PLL-coated 13-mm-diam coverslips in a volume of 50 μl per coverslip and also into two new flasks in a volume of 2 ml/flask. This passaging regime gave ~100 O-2A progenitor cells per coverslip. Flasks were grown for a further 10–14 d, after which the identical procedure was repeated. Cells on coverslips were stained after 1, 3, 7, and 10 d in culture.

Studies on Asymmetric Division and Differentiation

For experiments examining asymmetric division and differentiation, O-2A^{adult} progenitors isolated from optic nerves of >8-mo old rats (Wolswijk and Noble, 1989) were plated into slide flasks (Nunc, Roskilde, Denmark) containing confluent monolayers of irradiated (20 Grey) cortical astrocytes. Cells derived from one pair of adult optic nerves were plated into eight flasks; a plating density which gave 29 ± 8 A2B5$^+$GalC$^-$ O-2A^{adult} progenitors per flask. For examination of O-2A^{perinatal} progenitors, cells derived from the optic nerves of newborn rats were dissociated and plated into slide flasks at a density of 2,000 optic nerve cells per flask; this plating density yielded 32 ± 9 A2B5$^+$GalC$^-$ O-2A^{perinatal} progenitors per flask. After one day in vitro the flasks were examined with an inverted microscope and only those clusters containing well-isolated individual progenitor cells were retained for further analysis. [^{3}H]thymidine (2 μCi/ml) was added 24 h before staining of cultures with monoclonal A2B5 and anti-GalC antibodies, followed by appropriate conjugates (as in Noble and Murray, 1984; Wolswijk and Noble, 1989). Immunolabeling was carried out after 6, 8, and 10 d in culture for the perinatal cells and after 15 and 25 d in culture for the adult cells. Following immunolabeling, fixed cultures were dipped in a L-4 nuclear emulsion (Ilford, Knutsford, Cheshire, England), stored in the dark at 4°C for 48 h, and processed for autoradiography (as in Noble and Murray, 1984). Cultures were then examined with a microscope equipped with epi-illumination and fluorescein and rhodamine optics to identify colonies of immunolabeled cells. Groups of cells were only considered to be clones if they well isolated on the monolayers of cortical astrocytes. Clones containing oligodendrocytes were divided into three categories: (*a*) colonies that contained only GalC$^+$ oligodendrocytes; (*b*) colonies that contained oligodendrocytes and A2B5$^+$GalC$^-$ O-2A progenitors which were unlabeled with [^{3}H]thymidine; (*c*) colonies that contained oligodendrocytes and radiolabeled progenitors. Colonies lacking oligodendrocytes were further divided into two categories, according to whether or not members of the clone were labeled during a 20 h pulse with [^{3}H]thymidine. For presentation and analysis, data were pooled from several experiments. Single O-2A lineage cells were not included in this analysis.

Results

Generation of O-2A^{adult} Progenitors from O-2A^{perinatal} Progenitors

Analysis of the development of O-2A^{adult} progenitor cells in cultures derived from 3-wk-old rats, the age when the relative proportion of perinatal to adult O-2A progenitors appears to be changing most rapidly in vivo (Wolswijk et al., 1990), indicated that some O-2A^{perinatal} progenitor-like cells had the ability to generate O-2A^{adult} progenitor-like cells when co-cultured with purified cortical astrocytes. In seven individual time-lapse microcinematographic films, we identified 15 separate families of O-2A lineage cells suitable for detailed analysis (see Materials and Methods). Within these 15 families, we found four examples of families in which: (*i*) the founder cell gave rise at the first division to two cells with the characteristic morphology, cell-cycle length and motility of O-2A^{perinatal} progenitors; and (*ii*) subsequent to the first division, members of the family expressed the unipolar morphology, lengthened division times and slow migration rates typical of O-2A^{adult} progenitors.

Fig. 1 depicts diagrammatically the history of two of the families wherein O-2A^{perinatal} progenitor-like cells were seen to give rise to O-2A^{adult} progenitor-like cells, while Fig. 2 shows some of the actual images from the time-lapse films analyzed in these experiments. In family A the founder cell

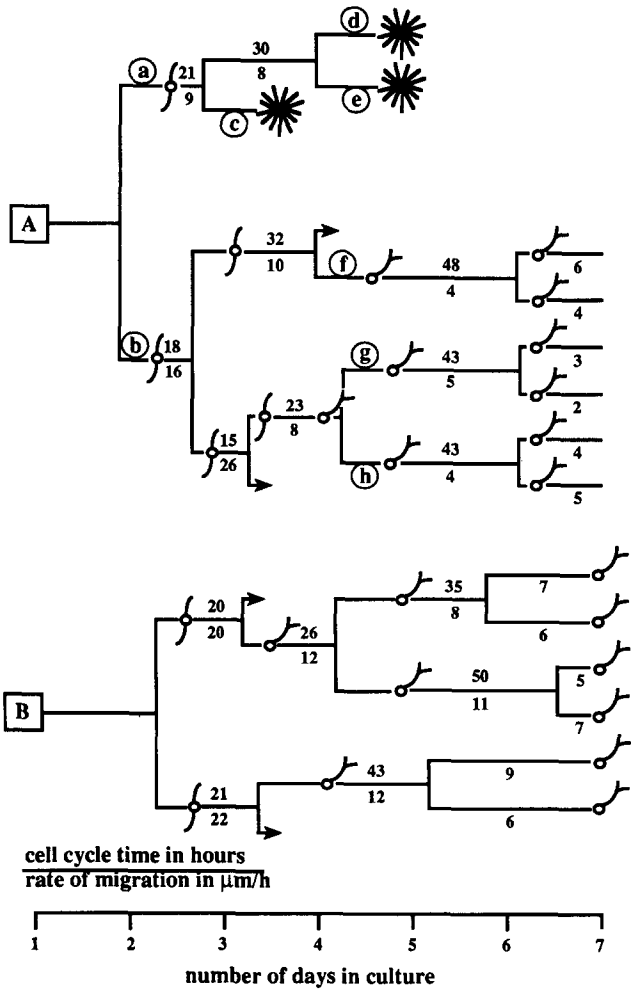


Figure 1. Bipolar O-2A^{perinatal} progenitor-like cells that divide and migrate at a fast rate give rise to unipolar O-2A^{adult} progenitor-like cells which migrate and divide more slowly. 15 clonal colonies of O-2A progenitors, stimulated to divide by cortical astrocyte-derived mitogens, were followed by time lapse microcinematography. Within 15 colonies suitable for detailed analysis, four clear examples were found of O-2A^{perinatal} progenitor-like cells that were bipolar, highly motile and had a short cell cycle time, which in the first division generated more O-2A^{perinatal} progenitor-like cells, and which eventually gave rise to cells which expressed the unipolar morphology, lengthened cell cycle time and slow migration rate of O-2A^{adult} progenitors. Two of the families in which dividing O-2A^{perinatal} progenitors gave rise to O-2A^{adult} progenitor-like cells are represented diagramatically in the figure. The morphology of a progeny cell is indicated in the figure only when the cell was clearly bipolar, unipolar, or oligodendrocyte-like. Since some progeny cells moved out of the field of photography (depicted with an arrow) their fate could not be determined. The numbers above the lines are the cell cycle times in hours, while the numbers below the lines are the migration rates in μm/h. The transitions shown could not be ascribed to changes in the composition of the tissue culture medium, since all cultures contained actively dividing and migrating O-2A^{perinatal} progenitor-like cells at the end of the filming period (see also Fig. 2).

first generated two O-2A^{perinatal} progenitor-like cells (cells *a* and *b* in Figs. 1 and 2). The family branch represented by one of these progenitors (cell *a* in Figs. 1 and 2) terminated, over two divisions, with the production of three oligodendrocytes (cells *c*, *d*, and *e* in Figs. 1 and 2, which were charac-

Table I. Changing Properties of Dividing O-2A Progenitor Cells with Serial Passage

Passage number	Percent of O-2A progenitors which were O4+	Percent of O-2A progenitors which were radiolabeled	Percent of radiolabeled cells which were A2B5+O4+
Start	0	85 ± 6	0
2	-	-	-
3	30 ± 17	23 ± 3	-
4	51 ± 2	23 ± 3	15 ± 9
5	77 ± 9	8 ± 1	70 ± 11
6	84 ± 2	9 ± 3	75 ± 10

	Number of colonies containing both GalC+ oligodendrocytes and radiolabeled A2B5+GalC- O-2A progenitor cells
3rd passage	10/52 (19%)
6th passage	9/29 (31%)

The properties of passaged O-2A progenitors change from *perinatal*-like to *adult*-like with increasing passage. Cultures initially devoid of O-2A^{adult} progenitors were grown on monolayers of purified cortical astrocytes, and serially passaged six times over the course of 3 mo (see Materials and Methods for experimental details). Passaged cells were then replated onto astrocyte monolayers in flasks or on coverslips, and cells on coverslips were analyzed for DNA synthesis, antigenic phenotype and morphology. (*Top*) In the beginning of the experiment, 85% of the O-2A progenitors were labeled with [^{3}H]thymidine, and none of these cells were O4+. By the 6th passage, only 9% of the O-2A progenitors were labeled by a 24-h pulse of [^{3}H]thymidine, and 75% of these cells were O4+. In addition, 84% of the entire O-2A progenitor population (defined as all A2B5+GalC- cells) was O4+. (*Bottom*) 31% of the colonies in the 6th passage contained oligodendrocytes and radiolabeled progenitor cells, as compared with 19% at the 3rd passage and 14% for unpassaged cells (see Fig. 4).

terized by their multipolar morphology, lack of division and lack of migratory behavior; Small et al., 1987; Noble et al., 1988). The other branch (cell *b*) first produced three further perinatal progenitor-like cells before all of these cells started to express longer cell cycle times and migration rates. By the next division, all of the motile and dividing members of this family expressed a unipolar morphology, a cell cycle length of >40 h ($\bar{x}$ = 45 h) and a migration rate of ≤6 μm/h ($\bar{x}$ = 4 μm/h); see cells *f*, *g*, and *h* in Figs. 1 and 2. In the second family (*B*) the first division produced two perinatal progenitor-like cells, after which all members of the family which remained within the field of visualization developed a unipolar morphology, slow migration rate ($\bar{x}$ = 8 μm/h) and lengthened cell-cycle times ($\bar{x}$ = 43 h), all characteristics of O-2A^{adult} progenitor cells. Similar observations were made in the other two families in which a perinatal-to-adult-transition was observed (data not shown).

Of the remaining 11 families analyzed, one family consisted of O-2A^{adult} progenitor-like cells from the initiation of filming. Ten families consisted of O-2A^{perinatal} progenitors which either continued to divide and migrate as *perinatal*-like cells throughout the filming period (see, for example, the cell marked with an open arrow in Fig. 2, *d* and *e*), or all observable members of the family differentiated into oligodendrocytes by the end of filming.

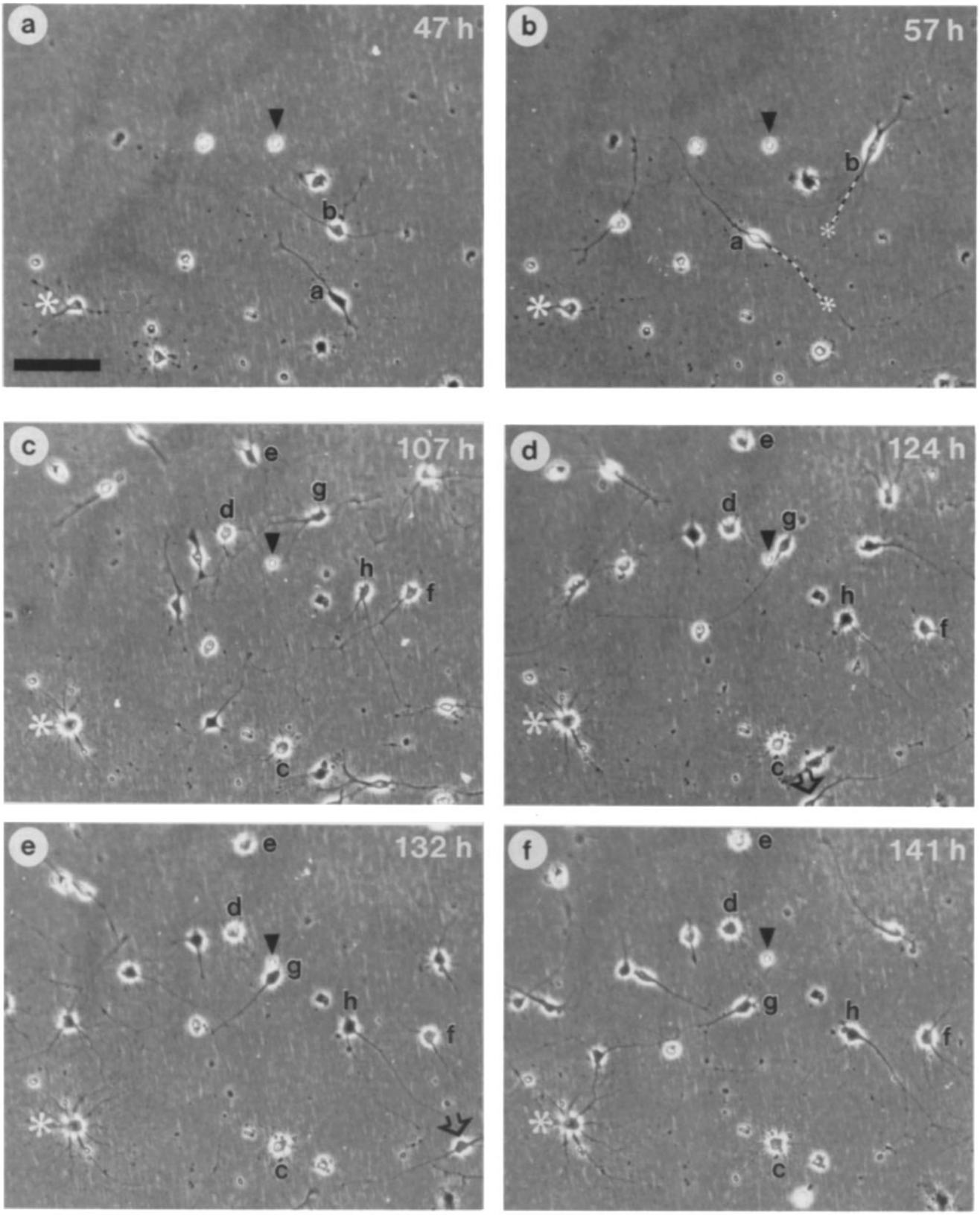
Extended Self Renewal in the O-2A Lineage Is Associated with the In Vitro Generation of O-2A^{adult} Progenitors

To test further the hypothesis that O-2A^{adult} progenitors might be derived from O-2A^{perinatal} progenitors, we serially passaged perinatal optic nerve cells over the course of 3 mo. In these experiments, optic nerve populations containing O-2A^{perinatal} progenitors, but not O-2A^{adult} progenitors (see Materials and Methods), were passaged onto fresh monolayers of purified and irradiated cortical astrocytes for up to six passages.

Serial passaging of O-2A progenitors derived from optic nerves of perinatal rats was associated with a shift in the progenitor population from entirely *perinatal*-like to predominantly *adult*-like, as judged by antigenic criteria, morphological criteria and changes in the population doubling times. In the early passage cultures, dividing O-2A progenitor-like cells (identified by [^{3}H]thymidine labeling, immunolabeling, and autoradiography) expressed the bipolar morphology and A2B5+O4- antigenic phenotype characteristic of O-2A^{perinatal} progenitor cells. In contrast, >80% of the dividing O-2A progenitors in the later passage cultures expressed the O4+ antigenic phenotype characteristic of O-2A^{adult} progenitors (Table I), and 92% of these cells also expressed the characteristic unipolar morphology of O-2A^{adult} progenitors (Wolswijk and Noble, 1989). The rate of increase in the numbers of new progenitors and oligodendrocytes in these cultures also decreased significantly with increasing passage number, and fell from the 24-h doubling times characteristic of perinatal populations to approach the long doubling times characteristic of adult populations (Fig. 3). In agreement with the increase in the average doubling time with increasing passage number, the proportion of progenitor-like cells which incorporated [^{3}H]thymidine during a 24-h pulse decreased with successive passages (Table I).

To confirm that passaged cells retained the capacity for normal differentiation, the passaged cells from one set of experiments were treated with anti-GalC antibody and complement (to eliminate GalC+ oligodendrocytes) after every passage before replating; new oligodendrocytes were consis-

Figure 2. Time-lapse microcinematographic demonstrations of the derivation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors. The cells shown correspond to the identically named cells in Fig. 1, family *A*. (*a* and *b*) Cells *a* and *b* expressed the bipolar morphology and rapid rate of migration of O-2A^{perinatal} progenitors. During the 10-h period between these two images, cells *a* and *b* migrated 100–150 μm. The dotted lines indicate their paths of migration. (*c–f*) Cells *c*, *d*, and *e* were all derived from cell *a*, and differentiated during the filming period into multipolar, non-dividing and non-migrating oligodendrocytes. (Oligodendrocytic differentiation also occurred in the cell marked with an asterisk in *a–f*, which was derived from a separate family of O-2A progenitors.) Cells *f*, *g*, and *h* were derived, within one to



two divisions, from cell *b* (see Fig. 1). Cells *f*, *g*, and *h* expressed the unipolar morphology, long cell cycle time and slow rate of migration characteristic of O-2A^{adult} progenitors. The cell marked with an open arrow in *d* and *e* expressed the characteristics of an O-2A^{perinatal} progenitor, illustrating that the generation of O-2A^{adult} progenitors did not represent a general conversion of all O-2A progenitors in the cultures. The debris marked with a closed arrow head, and the cell marked with an asterisk, offer constant reference points. The pictures shown represent ~30% of the entire field examined in this film. Bar, 100 μm.

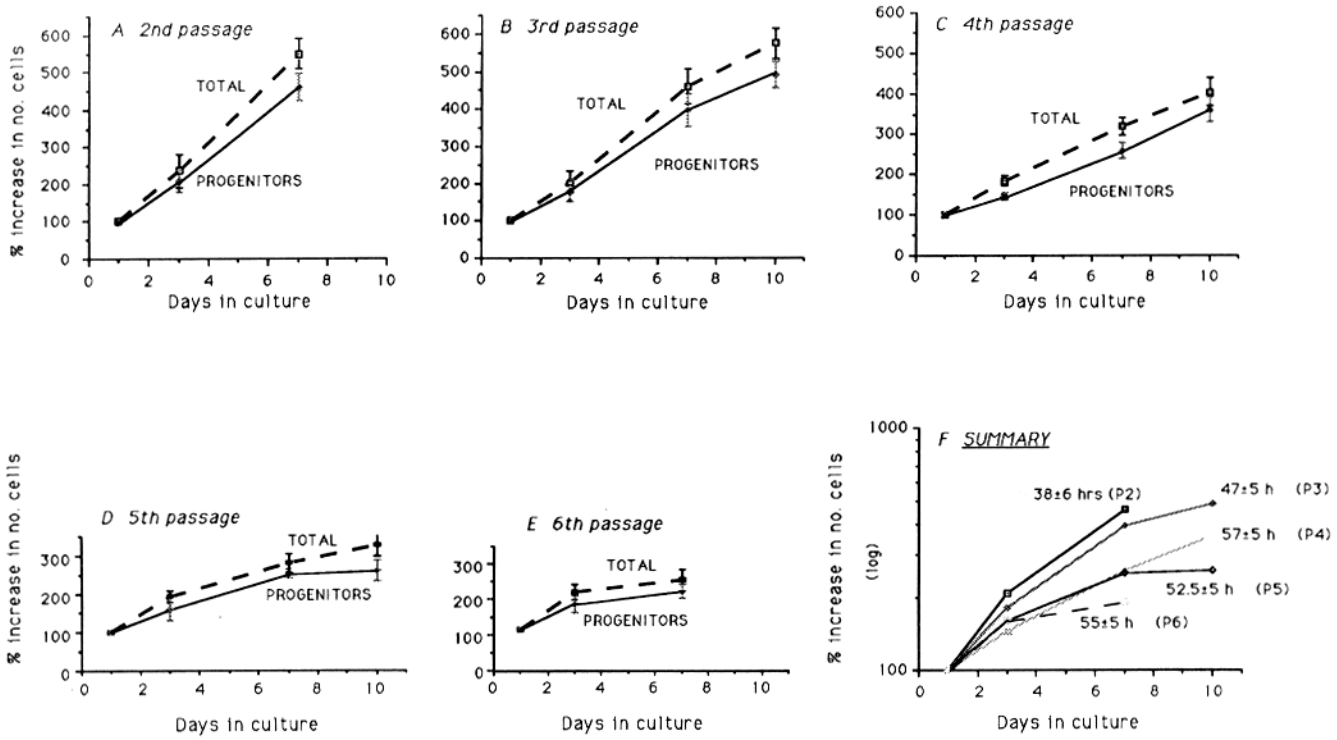


Figure 3. The rate of increase in the number of O-2A lineage cells (i.e., O-2A progenitors + oligodendrocytes) decreases with serial passaging in vitro. O-2A^{perinatal} progenitors were passaged through six passages on monolayers of purified cortical astrocytes. Passaged cells were replated on astrocyte monolayers both in flasks (for continued growth until subsequent passaging) and onto coverslips (for analysis). Cells on coverslips were immunolabeled to identify O-2A progenitors and oligodendrocytes, after which total numbers of each cell type were directly counted for each coverslip after 1, 3, 7, and 10 d in culture. The y axis is expressed as a percentage of the number of cells found on day one, in order to control for the inter-experimental variation in the number of O-2A progenitor cells available for replating after each serial passage. Each time-point is the mean ± SEM for triplicate coverslips from each of three separate experiments. The population doubling time for all O-2A lineage cells following each successive passage was 38 ± 6, 47 ± 5, 57.5 ± 5, 52.5 ± 4, and 55 ± 5 h for the 2nd through 6th passages, respectively (*F*). The doubling time for the starting population was 22 ± 5 h (not shown), in agreement with previous characterization of cell cycle time of the perinatal progenitors. The number of oligodendrocytes is equal to the number of total O-2A lineage cells minus the number of O-2A progenitors.

tently generated in these cultures when cells were grown on monolayers of cortical astrocytes. In addition, the numbers of oligodendrocytes always increased with time in cultures generated after each serial passaging (see Fig. 3). Moreover, type-2 astrocytes were produced when cells were replated in medium containing fetal calf serum (as in Raff et al., 1983*b*; Wolswijk and Noble, 1989; data not shown).

Lysis with A2B5 and anti-GalC antibodies and complement completely eliminated all O-2A lineage cells (data not shown), in agreement with our other observations indicating that O-2A lineage cells were only derived from either O-2A^{perinatal} or O-2A^{adult} progenitors, both of which are A2B5$^+$. In addition, passage of purified and irradiated astrocytes which had not received optic nerve cells yielded no cells of the O-2A lineage, confirming that the newly arising cells in our passaging experiments were not derived from the astrocyte monolayers.

We also noted that serial passaging was associated with a slight increase in the proportion of colonies which contained both oligodendrocytes and [^{3}H]thymidine labeled O-2A progenitors (Table I), an observation examined in closer detail using cells derived from adult animals (see next section).

Characteristics of Division and Differentiation in Colonies of O-2A^{adult} Progenitor Cells

As cultures derived from optic nerves of 1-mo-old rats contain few O-2A^{perinatal} progenitors, and such cells are not detected at all in cultures derived from adult optic nerves, it is unlikely that generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors is the mechanism which allows maintenance of the *adult* progenitor in the nerve throughout life. The slight increase in the proportion of O-2A lineage colonies containing both oligodendrocytes and [^{3}H]thymidine-labeled O-2A progenitors, seen in our passaging experiments, raised the possibility O-2A^{adult} progenitors might be able to divide and differentiate asymmetrically. Such a pattern of division and differentiation would allow these cells to give rise to more progenitors at the same time as they generated oligodendrocytes. To examine this possibility under conditions which would allow cells to undergo several divisions, we analyzed the composition and size of oligodendrocyte-containing colonies generated from O-2A^{perinatal} and O-2A^{adult} progenitors grown at clonal densities (<1 cell/30 mm^2) on monolayers of purified cortical astrocytes (to promote progenitor division; Noble and Murray, 1984; Wol-

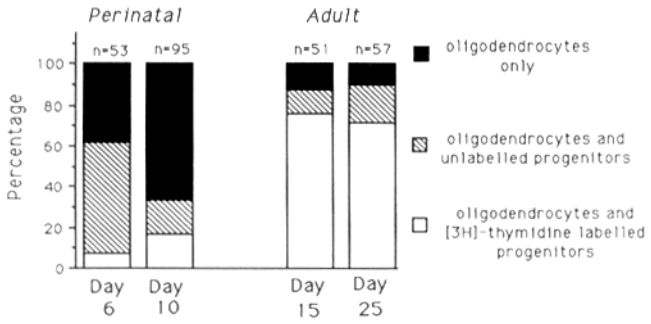


Figure 4. Oligodendrocyte-containing colonies derived from O-2A^{adult} progenitors also tend to contain [^{3}H]thymidine-labeled progenitor cells, while similar colonies derived from O-2A^{perinatal} progenitors rarely contain dividing progenitors. Clonal colonies, growing on monolayers of purified cortical astrocytes, were pulsed with [^{3}H]thymidine for 24 h before immunostaining and autoradiography. The composition of each clonal colony was then determined. In this figure, only data from colonies containing oligodendrocytes is presented. There is a highly significant different ($p << 0.001$; all χ^2 values > 49.5) between the perinatal and adult colonies at all time points, but not between the adult colonies at the two time points ($\chi^2 = 0.85$, $p > 0.5$). The results shown were obtained from three separate experiments each with two slide flasks per time point.

swijk and Noble, 1989). As the generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors (as would occur in cultures derived from 3-wk-old rats) would have complicated analysis of these experiments, the optic nerve cells used in these experiments were obtained exclusively from newborn and adult rats. Colonies were examined after a length of time which would allow cells to undergo ≤6 divisions and ≤10 divisions, this being 6 and 10 d for O-2A^{perinatal} progenitors and 15 and 25 d of O-2A^{adult} progenitors, respectively.

Oligodendrocyte-containing Colonies

As in previous experiments (Temple and Raff, 1986), the composition and size of oligodendrocyte-containing colonies derived from O-2A^{perinatal} progenitors were consistent with the view that the generation of oligodendrocytes by these cells is associated with symmetric division and clonal differentiation (Figs. 4 and 5). 66% of the oligodendrocyte-containing colonies examined on day 10 consisted entirely of oligodendrocytes and, even as early as day 6, the A2B5$^+$GalC$^-$ progenitor-like cells in mixed colonies were most frequently multipolar nondividing cells (i.e., not labeled with [^{3}H]thymidine) which appeared to have been visualized just before oligodendrocytic differentiation. Only 7% of the oligodendrocyte-containing colonies derived from *perinatal* progenitors and visualized on day 6, and 14% of those visualized on day 10, contained both oligodendrocytes and dividing progenitor cells (as judged by the incorporation of [^{3}H]thymidine; Fig. 4). Moreover, oligodendrocyte-containing colonies derived from O-2A^{perinatal} progenitors clustered around sizes of 2, 4, 8, 16, 32, 64, and 128 cells/colony at 6, 8, and 10 d after plating (Fig. 5), as expected when clonally related cells divide symmetrically and differentiate synchronously.

Unlike the results obtained with O-2A^{perinatal} progenitors, the composition of oligodendrocyte-containing colonies derived from individual dividing O-2A^{adult} progenitors was

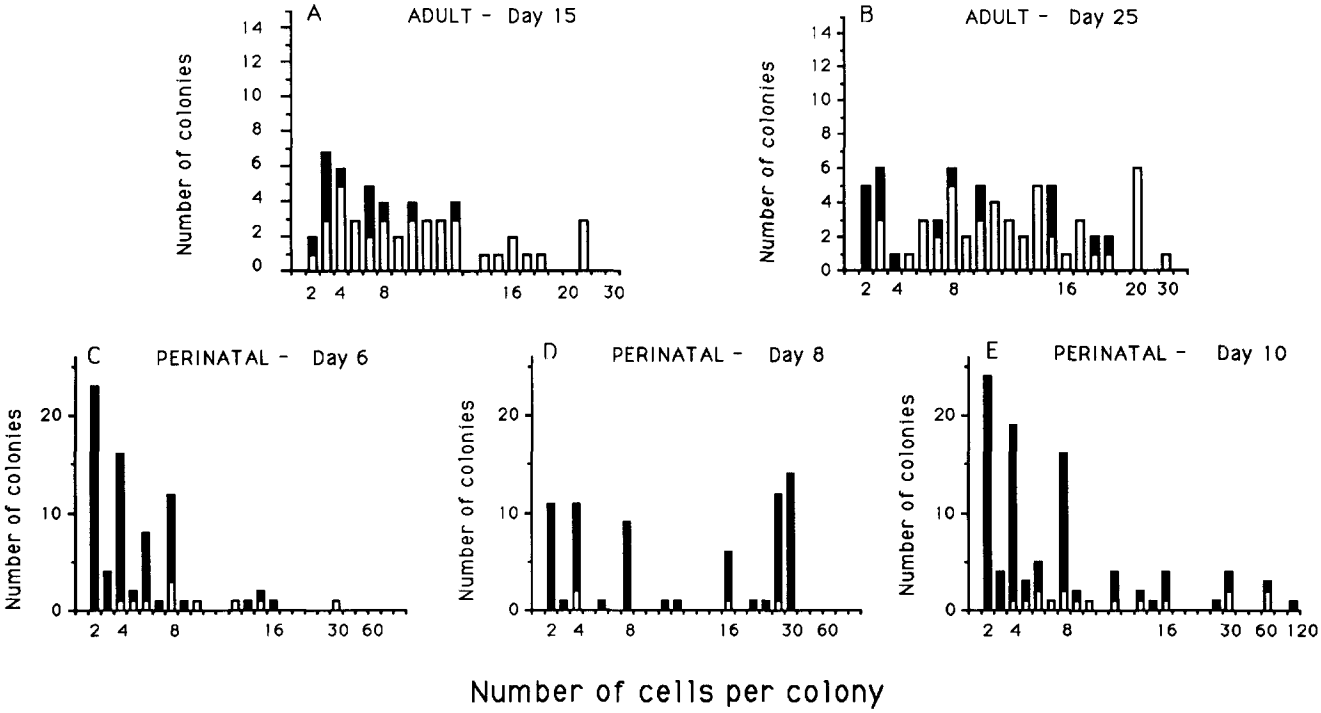


Figure 5. The size of oligodendrocyte-containing colonies derived from O-2A^{adult} progenitors do not cluster around powers of two (*A* and *B*), while oligodendrocyte-containing colonies derived from O-2A^{perinatal} progenitors cluster at sizes of 2, 4, 8, 16, 32, 64, and 128 (*C–E*). (□) Colonies containing both oligodendrocytes and radiolabeled progenitors. (■) Represent oligodendrocyte-containing colonies in which there were no dividing progenitor cells. The colonies analyzed are identical to those analyzed for their cell type composition in Fig. 4.

consistent with the hypothesis that the generation of oligodendrocytes by these cells occurred by means of asymmetric division and differentiation (Fig. 4). Over 75% of the oligodendrocyte-containing colonies derived from individual O-2A^{adult} progenitors grown at clonal densities contained both oligodendrocytes (which generally do not divide in these tissue culture conditions; Noble and Murray, 1984; Wolswijk and Noble, 1989) and [^{3}H]thymidine-labeled progenitors after both 15 and 25 d of in vitro growth (Fig. 4), periods of time which would allow ≤6 or ≤10 average cell cycles for O-2A^{adult} progenitors (Wolswijk and Noble, 1989). The proportion of colonies which contained both oligodendrocytes and radiolabeled O-2A progenitor cells on days 15 and 25 of in vitro growth (Fig. 4) was very similar, even though the average size of the oligodendrocyte-containing colonies continued to increase with time in culture (from a median value of seven cells/colony on day 15 to a medium value of 11 cells/colony on day 25; Fig. 5). Only 10% of the colonies visualized on day 25 consisted entirely of oligodendrocytes, and the remaining 14% contained oligodendrocytes and progenitors which were unlabeled by [^{3}H]thymidine. In addition, sizes of oligodendrocyte-containing colonies did not cluster at factors of two on either day 15 or day 25 of in vitro growth (Fig. 5).

Oligodendrocyte-free Colonies

As shown in Table II, 62% (110/176) of the colonies derived from O-2A^{adult} progenitors contained no oligodendrocytes even after 25 d of in vitro growth. Oligodendrocyte-free colonies seen at this stage were generally small, and >80% of these colonies (89/110) contained ≤16 cells at day 25 (Fig. 6). Consistent with the small size of many of these colonies, <20% (23/110) of the oligodendrocyte-free colonies contained any cells which were labeled by a 20-h pulse with [^{3}H]thymidine. In contrast, in colonies derived from O-2A^{perinatal} progenitors, only 30% (41/136) of the colonies were free of oligodendrocytes on day 10 in vitro. In contrast to the colonies derived from O-2A^{adult} progenitors, [^{3}H]thymidine labeled progenitors were found in 80% (33/41) of all the oligodendrocyte-free colonies derived from O-2A^{perinatal} progenitors, regardless of colony size, and were even found in 75% (12/16) of the colonies containing four cells or less.

Table II. Composition of O-2A^{adult} Progenitor-derived Colonies

	Perinatal day 10	Adult day 25
Analysis of all colonies		
Colonies with oligodendrocytes	95 (70%)	66 (38%)
Oligodendrocyte-free colonies	41 (30%)	110 (62%)
Analysis of oligodendrocyte-free colonies		
	Perinatal day 10	Adult day 25
Colonies with [^{3}H]thymidine labeled cells	33 (80%)	23 (21%)
Colonies with no [^{3}H]thymidine labeled cells	8 (20%)	87 (79%)

(*Top*) Most colonies derived from O-2A^{adult} progenitors contain no oligodendrocytes, even after 25 d in culture. In contrast, most colonies derived from O-2A^{perinatal} progenitors contain oligodendrocytes after 10 d in culture. Clonal colonies of O-2A progenitors derived from optic nerves of perinatal or adult rats were grown on monolayers of cortical astrocytes for the indicated time period, and then pulsed with [^{3}H]thymidine for 20 h and labeled with A2B5 and anti-GalC antibodies to allow identification of cell-types of interest. (*Bottom*) In most of the oligodendrocyte-free colonies derived from O-2A^{adult} progenitors, no progenitor cells were labeled by a 20-h pulse with [^{3}H]thymidine. In contrast, there were radiolabeled cells in most of the colonies derived from O-2A^{perinatal} progenitors. See text for more details, and Materials and Methods for experimental details.

Discussion

We have addressed several aspects of the biology of the O-2A^{adult} progenitor, the only glial precursor cell which thus far has been identified unambiguously in cultures derived from adult CNS tissue. We have provided evidence indicating that O-2A^{adult} progenitors are derived from a subpopulation of O-2A^{perinatal} progenitors in vitro. We also found that O-2A lineage cells are able to undergo continuous self renewal through at least 3 mo of serial passaging in vitro, and that serial passaging of O-2A^{perinatal} progenitors is associated with the generation of O-2A^{adult} progenitors. In addition, we provide evidence suggesting that the generation of oligodendrocytes from O-2A^{adult} progenitors occurs primarily through asymmetric division and differentiation, and thus differs from the symmetric division and differentiation seen in families of O-2A^{perinatal} progenitors. Finally, analysis of colonies derived from O-2A^{adult} progenitors suggests that this population also contains relatively quiescent cells that are able to survive for extended periods in vitro while undergoing little division or differentiation.

Our time-lapse microcinematographic observations of cultures of dividing O-2A^{perinatal} progenitors showed directly that cells expressing the bipolar morphology, short cell cycle time and rapid motility of 0-2A^{perinatal} progenitors could give rise to cells which expressed the unipolar morphology, long-cell cycle times and slow rates of migration characteristic of O-2A^{adult} progenitors. Although the interpretation of these experiments is dependent upon the correct morphological identification of these two cell types, our previous studies (Noble and Murray, 1984; Noble et al., 1988; Raff et al., 1988; Wolswijk and Noble, 1989; Wolswijk et al., 1990, 1991) have repeatedly failed to identify any other cells in these cultures which express such characteristics. Our own studies, and other recent studies on a pre-O-2A progenitor cell found in cultures of cerebral cortex (Grinspan et al., 1990) have also failed to find any developmental ancestor for O-2A progenitors in the optic nerve other than O-2A progenitors themselves. In addition, in all of our observations on cells derived from adult rats, we have only ever observed generation of O-2A^{adult} progenitors from other cells with the characteristics of O-2A^{adult} progenitors (G. Wolswijk, unpublished observations).

It was striking that in all of the O-2A^{perinatal} progenitor families which gave rise to *adult* progenitor-like cells, the appearance of O-2A^{adult} progenitors occurred in all branches of the family tree which still contained proliferating cells (Fig. 1 and unpublished observations), even though the culture dishes still contained other O-2A^{perinatal} progenitors. Thus, the appearance of the O-2A^{adult} progenitor phenotype appears to be a specific property expressed by individual families of cells, rather than being the result of, e.g., the sudden appearance in culture of a factor(s) which induces such a change in all O-2A^{perinatal} progenitors. This interpretation is consistent with other observations indicating that O-2A^{adult} and O-2A^{perinatal} progenitor can co-exist in vitro and in vivo (Wolswijk et al., 1990). Moreover, our time-lapse observations suggest that the transition from *perinatal* to *adult* phenotype is not an abrupt one, in that generation of O-2A^{adult}

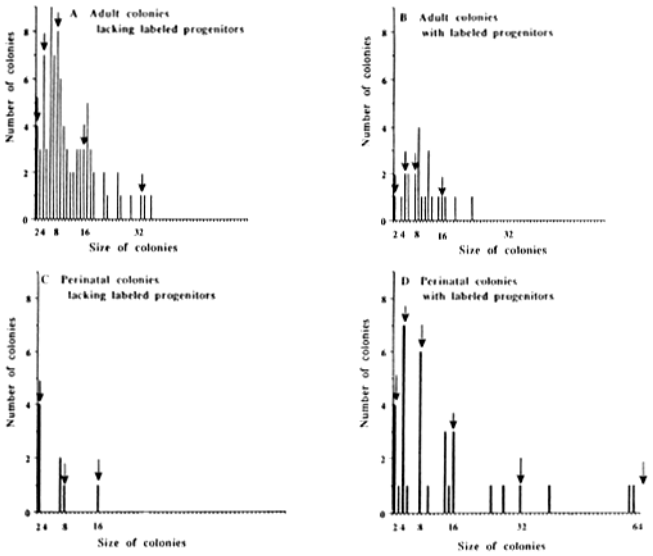


Figure 6. The distribution of colony sizes for oligodendrocyte-free colonies derived from O-2A^{adult} or O-2A^{perinatal} progenitors shows that most of the colonies derived from O-2A^{adult} progenitors were of sizes consistent with average cell cycle times in excess of 75 h, and a significant proportion of colonies were of sizes consistent with average cell cycle times in excess of 150 h. The colonies analyzed in this figure are from the same experiments as for Fig. 4 and 5, but this figure presents data from colonies lacking oligodendrocytes. The distribution of colony sizes also suggests that division was more highly synchronized in colonies derived from O-2A^{perinatal} progenitors than in colonies derived from O-2A^{adult} progenitors, as only the *perinatal* progenitor-derived colonies were clustered around factors of two in size. Finally, the majority of oligodendrocyte-free colonies derived from O-2A^{perinatal} progenitors contained cells which took up [^{3}H]thymidine during a 20-h pulse, while only a minority of colonies derived from *adult* progenitors contained cells which were labeled with such a pulse. The arrows indicate those colonies whose sizes were equal to factors of two. See text for more details, and Materials and Methods for experimental details.

progenitor-like cells may require two or more cell divisions, with the cells present after one division expressing cell cycle times and motility characteristics intermediate between the *perinatal* and *adult* phenotypes. These results also are consistent with our previous studies on the characteristics of cells derived from optic nerves of 1-wk– 1-mo-old rats, in which we observed cells with phenotypes which could not be classified unambiguously as *adult*-like or *perinatal*-like (Wolswijk et al., 1990).

While the generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors provides a possible explanation for the origin of the *adult* cell, the lack of O-2A^{perinatal} progenitors in adult optic nerve (Wolswijk and Noble, 1989) suggests that other mechanisms are involved in maintenance of the O-2A^{adult} progenitor population in the adult animal. Although it has been previously suggested (ffrench-Constant and Raff, 1986) that the presence of such cells in the adult requires the existence of an ancestral stem cell, capable of generating O-2A lineage cells throughout life, several observations now raise the possibility that the O-2A^{adult} progenitors may themselves function as stem cells.

The first stem cell-like property of O-2A^{adult} progenitors derives from the observation that this population is maintained in the rat optic nerve as a dividing population seemingly throughout life (ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989; G. Wolswijk and E. Abney, unpublished observations). O-2A^{adult} progenitor-like cells can be isolated from optic nerves during the first week after birth (Wolswijk et al., 1990) and such cells remain in the nerve for at least the first year of life, in contrast with O-2A^{perinatal} progenitors, which have largely disappeared from the optic nerve by one month after birth. In vitro observations suggest that O-2A^{perinatal} progenitors would disappear from the nerve as a consequence of symmetric differentiation of most clones of cells into oligodendrocytes and differentiation of the remaining cells into O-2A^{adult} progenitors (and possibly type-2 astrocytes, although the in vivo existence of these cells is controversial; e.g., Barres et al., 1990; Richardson et al., 1990; Noble, 1991; Skoff and Knapp, 1991).

The second stem cell-like property of O-2A^{adult} progenitors is their long (60–65 h) cell cycle times (Wolswijk and Noble, 1989; Wolswijk et al., 1990, 1991). Our present studies further suggest that the population of O-2A^{adult} progenitors may even contain a small proportion of cells with cell cycle times in excess of 100 h. Examination of colonies developing in vitro over 25 d showed that the great majority (89/110) of these colonies contained ≤16 cells after 25 d in vitro, and that only a small proportion (23/110) of these colonies contained cells which were labeled with a 20-h pulse of [^{3}H]thymidine. Both of these results are consistent with the existence of O-2A^{adult} progenitors with very long cell cycle times. In contrast, it was surprising to find that 30% of the colonies derived from O-2A^{perinatal} progenitors in these particular experiments were of small size and contained no oligodendrocytes after 10 d of in vitro growth. Yet, despite their small size, 75% of these oligodendrocyte-free colonies contained progenitors labeled with a 20-h pulse of [^{3}H]thymidine, suggesting that these cells were dividing with a frequency expected for O-2A^{perinatal} progenitors. As O-2A^{perinatal} progenitors are extensively migratory in vitro it may be these small colonies were derived from *perinatal* progenitors which migrated a sufficient distance from their origin so as to be scored as a distinct colony.

Our observations consistent with the view that O-2A^{adult} progenitors can undergo asymmetric division and differentiation in vitro are also of potential relevance to the question of whether these cells express stem cell-like characteristics. Unlike colonies derived from O-2A^{perinatal} progenitors, oligodendrocyte-containing colonies derived from O-2A^{adult} progenitors generally also contained O-2A^{adult} progenitors which were labeled by [^{3}H]thymidine, indicating that onset of differentiation in the *adult* progenitor-derived colonies was not associated with cessation of cell division in the whole colony. These results could have been a result of asymmetric division and differentiation or, alternatively, to asynchronous division rates in different branches of a family tree, but with each branch in the family still limited to an identical total number of divisions. The latter explanation seems unlikely for two reasons: First, if the mixed composition of the colonies was because of a failure of some branches of the family trees to reach their allotted number of divisions within the first 15 d of in vitro growth, we then would have expected to see an increased proportion of colonies containing only oligodendrocytes, or oligodendrocytes and unlabeled progenitors, between days 15 and 25 (Fig. 3). Instead, the proportion of colonies containing both oligodendrocytes and [^{3}H]thymidine-labeled progenitors at days 15 and 25 was essentially identical. In addition, if differentiation within a family was still symmetric (as for *perinatal* progenitors), then we would have expected to see colonies lacking dividing adult progeni-

tors to cluster at factors of two in size. No such clustering was apparent. It is also unlikely that these results were due to migration of O-2A^{adult} progenitors to form mixed colonies, as these cells migrate very slowly on astrocyte monolayers (~2 μm/h; Wolswijk and Noble, 1989); the potential of producing such artifacts from cell migration would be expected to be far greater in cultures containing rapidly migrating O-2A^{perinatal} progenitors. There also is no obvious way that cell death could be responsible for production of the colonies containing oligodendrocytes and [^{3}H]thymidine-labeled progenitors. Thus, our data suggests that generation of oligodendrocytes from O-2A^{adult} progenitors in these experiments occurred predominantly though asymmetric division and differentiation.

A further stem cell-like feature displayed by *adult* progenitors was that a far higher proportion of oligodendrocyte-containing colonies than oligodendrocyte-free colonies contained O-2A^{adult} progenitors labeled with a 20-h pulse of [^{3}H]thymidine (75 vs 20%; Fig. 4 and Table II). Similarly, the onset of differentiation of epidermal stem cells into keratinocytes in any clone of cells is associated with an increased likelihood of finding cells engaged in DNA synthesis, in association with passage of stem cell progeny through a transit amplifying population of cells engaged in differentiation (Hall and Watt, 1989).

The results of our serial passaging experiments also were consistent with the hypotheses that O-2A^{adult} progenitors are derived from O-2A^{perinatal} progenitors and that expression of the capacity for prolonged self-renewal in this lineage is associated with the appearance of O-2A^{adult} progenitors. In contrast, if these experiments had revealed a lack of capacity for prolonged self renewal, or the failure of passaged populations to generate O-2A^{adult} progenitors, this would have suggested the hypotheses presented are not correct. It is interesting that this replacement of an O-2A^{perinatal} progenitor population by an O-2A^{adult} progenitor population in vitro is at least superficially similar to that which occurs in vivo (albeit over a slightly shorter time scale than that seen in vitro). The ability to reproduce such a conversion in tissue culture will facilitate future studies on molecular mechanisms which might be involved in the generation of O-2A^{adult} progenitors from O-2A^{perinatal} progenitors.

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